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Supplemental Information

A Chaperone Lid Ensures Efficient and Privileged Client Transfer during Tail-Anchored Protein Targeting

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Supplemental Information

Inventory of Supplemental Information

15 **Supplemental Figures and Figure Legends**

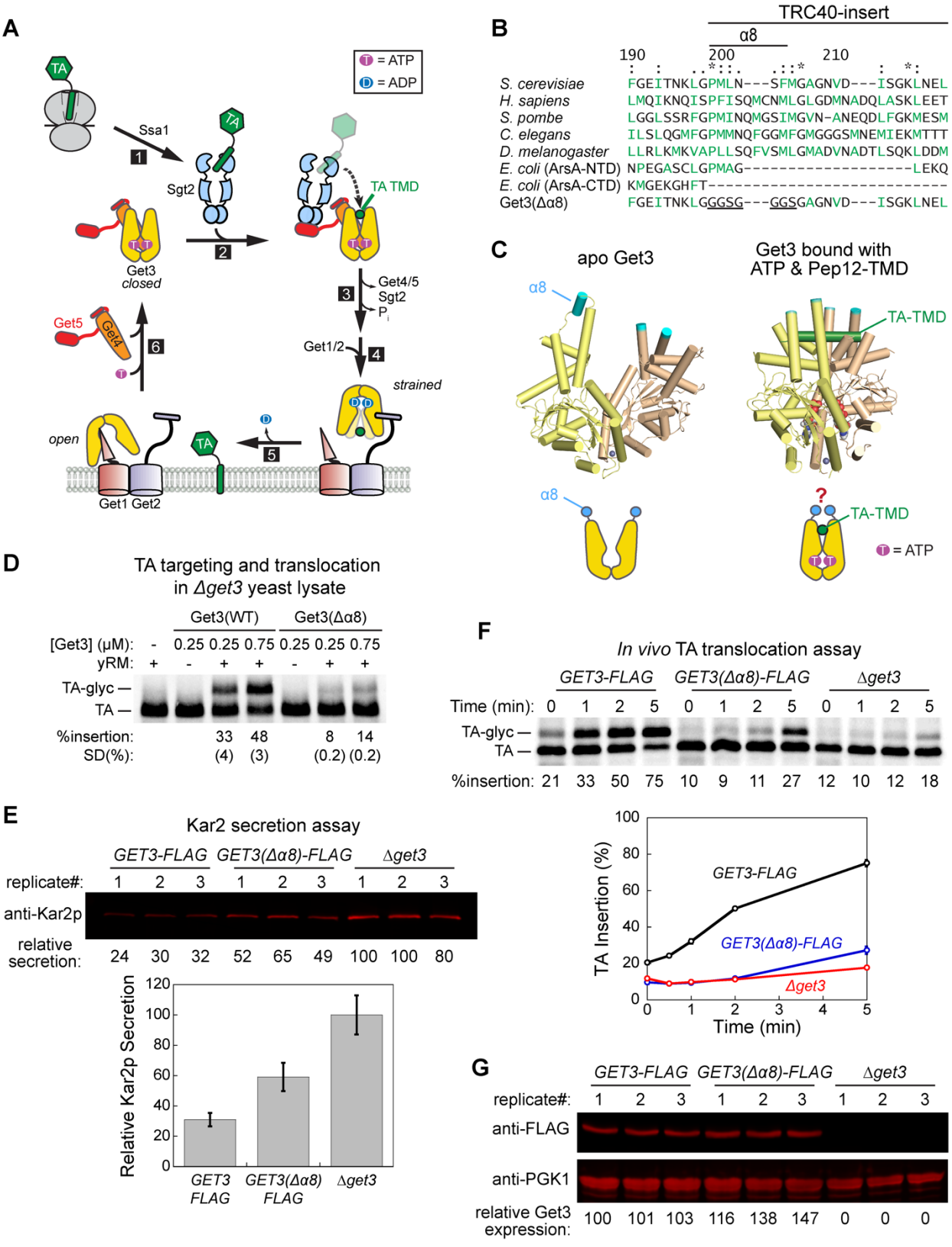

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Figure S1. A conserved α8 motif in Get3 is important for TA targeting to the ER. Related to Figures 1, 2, and 3.

(A) Current model of the GET pathway in yeast. Newly synthesized TAs from the ribosome are first captured and chaperoned by Ssa1 and then delivered to the co-chaperone Sgt2 (step 1). Sgt2 next delivers the TA to the ATPase Get3 (step 2), which in the cytosol is ATP-loaded and bound to a scaffolding complex Get4/5 that also interacts with Sgt2. ATP and Get4/5 pre-organize Get3 in a closed conformation, and Get4/5 further inhibits Get3's ATPase activity to prime Get3 for TA capture. TA loading onto Get3 induces Get3 to sample more open conformations that drives Get4/5 dissociation and activates Get3 to hydrolyze ATP (step 3). The resulting Get3•TA complex is in a strained conformation primed to interact with the Get1/2 membrane receptors (step 4). Get1/2 disassembles the Get3•TA complex and facilitates TA insertion into the ER (step 5), leaving Get3 bound to Get1 in an open conformation. ATP and Get4/5 help release Get3 from Get1 and recycle Get3 for further rounds of targeting (step 6).

(B) Sequence alignment of α8 and its flanking sequences among Get3 homologues. The mutations introduced in Get3(Δα8) are also shown (underlined). Hydrophobic residues are highlighted in green. '.' and ':' denote residues that are modestly or highly conserved in amino acid characteristic, respectively, and '*' denotes residues that are highly conserved in identity.

(C) The α8 motif and adjacent residues are highlighted (cyan) in the crystal structures of Get3 in the *open* (left; PDB 3H84) and *closed* (right; PDB 4XTR) conformations. Only one α8 motif is

resolved in the *open* structure, while neither α8 motifs are not resolved in the *closed* structure. The question mark ("?") denotes the uncertainty of the positioning of the α8 motifs.

(D) TA targeting and translocation by wild-type Get3 or mutant Get3(Δα8) in the *Δget3* yeast lysate. 10 μL translation reactions for the model TA Bos1 were carried out in *Δget3* lysate in the presence of 10 μCi [^{35}S]-methionine and purified Get3 or Get3(Δα8) at indicated concentrations. After 30 min at 26 °C, cyclohexamide was added to stop translation. *Δget3* microsomes were added where indicated, and the reactions were incubated for 40 min at 26 °C and quenched by SDS sample buffer. Samples were analyzed by SDS-PAGE and autoradiography. Insertion efficiency was calculated using Equation 2.

(E) Kar2p secretion by *GET3-FLAG*, *GET3(Δα8)-FLAG*, and *Δget3* yeast cells, measured as described (Schuldiner et al., 2005). Yeast cultures were grown to saturation in YPD at 30 °C overnight and diluted to an OD_{600} of 0.1 in fresh YPD. Cultures were grown at 30 °C to mid-log phase (OD_{600} ~ 1) and harvested. Cells were resuspended in fresh YPD to an OD_{600} of 0.5 and incubated at 30 °C for 2 hours. Cells were harvested, and proteins from 1.35 mL of the supernatant (media) was precipitated by 10% TCA, washed with acetone, resuspended in 35 μL of SDS sample buffer, and neutralized by titrating 1M Tris-HCl pH 9.5. Samples were analyzed by Western blot using an anti-Kar2 antibody (gift from P. Walter) at 1:3000 dilution. IRDye® 800CW Goat anti-Rabbit secondary antibody (LI-COR Biosciences) at 1:20,000 dilution was used for visualization using an Odyssey infrared imaging system. The top panel shows a representative western blot. The bottom panel shows quantification of the relative amount of secreted Kar2p from three biological replicates. Values are reported as mean ± SD.

(F) Pulse-chase analysis to monitor the translocation of newly synthesized TA *in vivo*, carried out as described in STAR Methods (Cho and Shan, 2018). Insertion into the ER results in glycosylation of the opsin tag on the TA, which allows untranslocated TA and glycosylated TA (TA-glyc) to be resolved on SDS-PAGE (top panel). The bottom panel shows the quantification of three independent experiments. The values represent mean ± SD (3 biological replicates). Error bars are shown but may be too small to be visible.

(G) Clarified lysates of the cells from the same cultures in (D) were diluted with SDS sample buffer and immunoblotted against an anti-FLAG antibody (GenScript; top) to determine the steady state Get3 levels in the different yeast strains. An anti-PGK1 (Abcam) blot served as a control for normalization (bottom). IRDye® 800CW Goat anti-Mouse secondary antibody (LI-COR Biosciences) was used for visualization using an Odyssey imaging system. Get3(Δα8)-FLAG is slightly overexpressed in *GET3(Δα8)-FLAG* cells compared to *GET3-FLAG* cells.

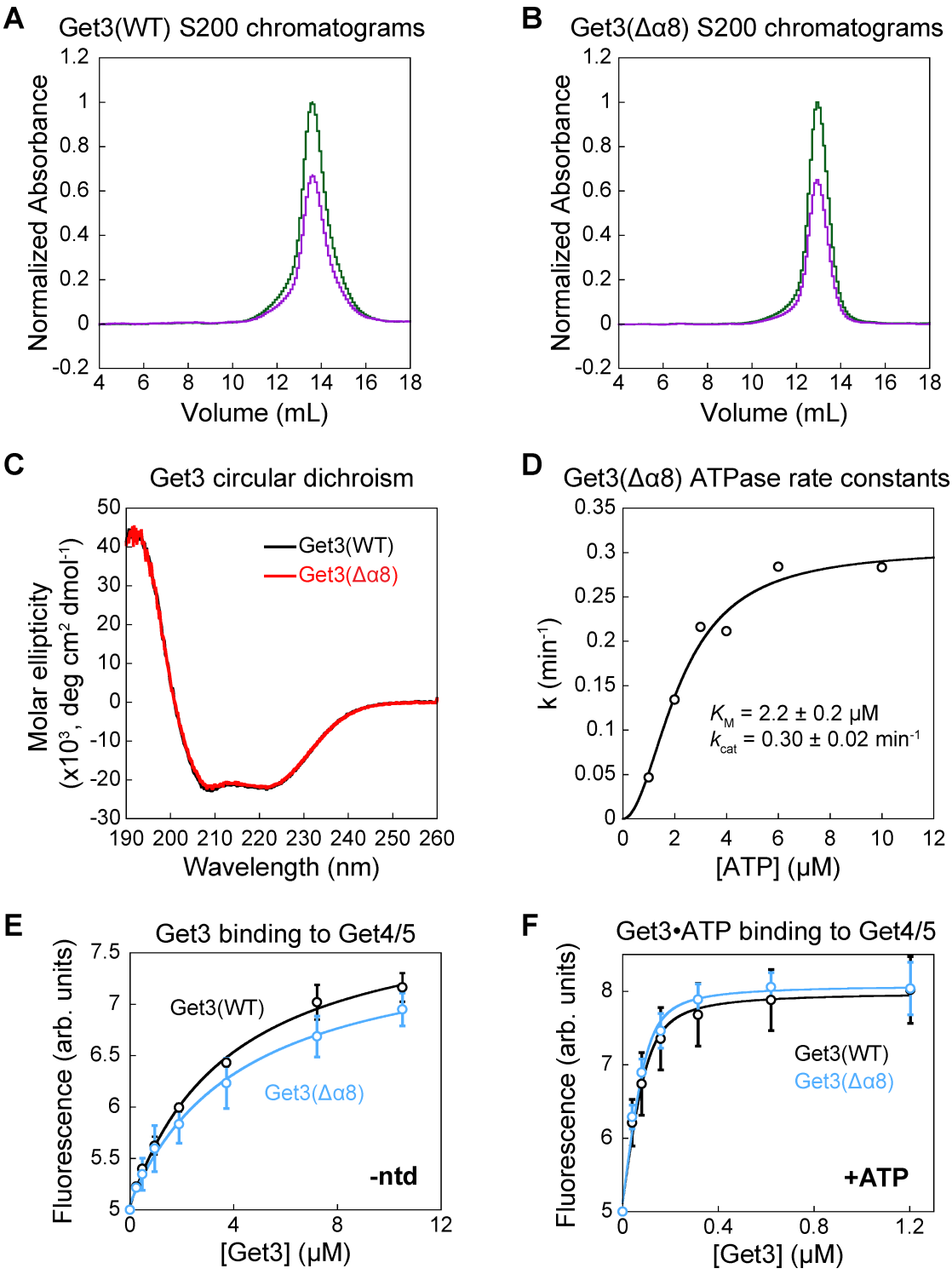


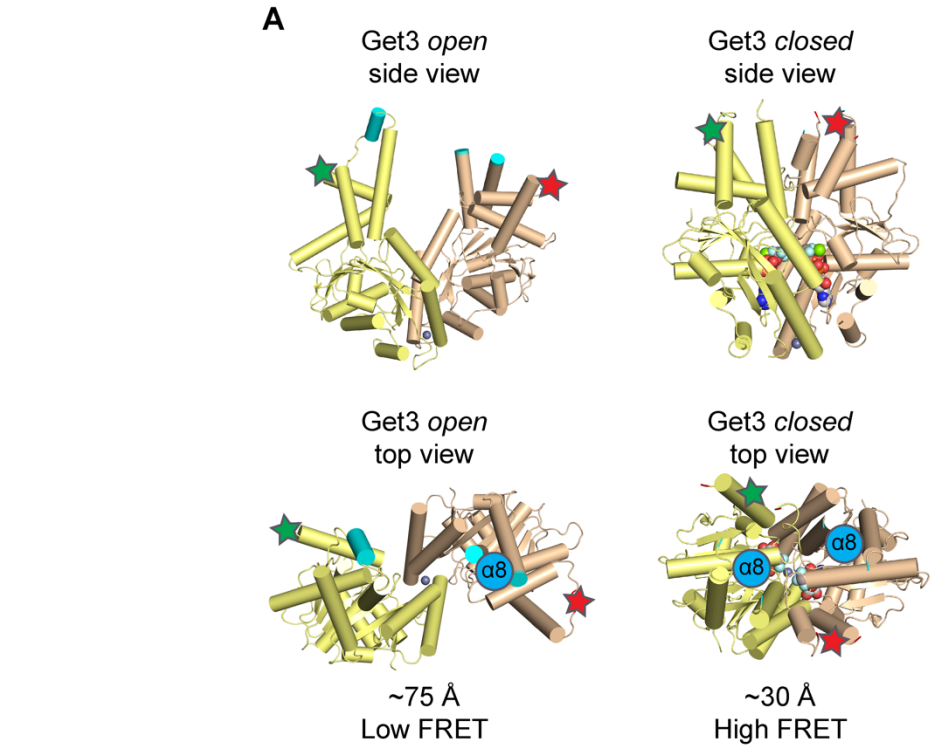
Figure S2. Get3(Δα8) exhibits the same global structure, basal ATPase activity, and ATP-dependent binding to Get4/5 as wild-type Get3. Related to Figures 1, 2, and 3.

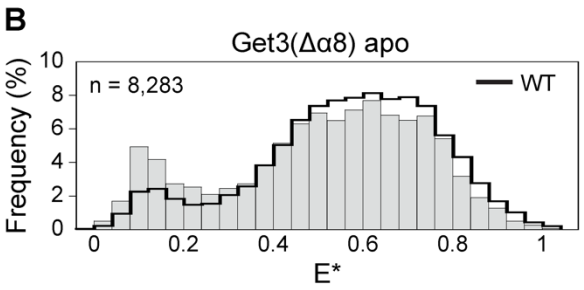
(A), (B) Size-exclusion chromatogram of purified wild-type Get3 (A) and mutant Get3(Δα8). The green and purple lines denote A_{280} and A_{260}, respectively.

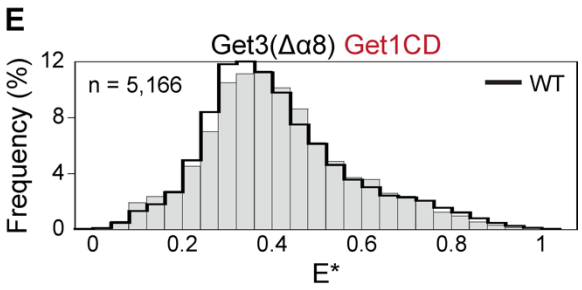
(C) Circular dichroism spectra of 5 μM Get3(WT) (black) and 5 μM Get3(Δα8) (red).

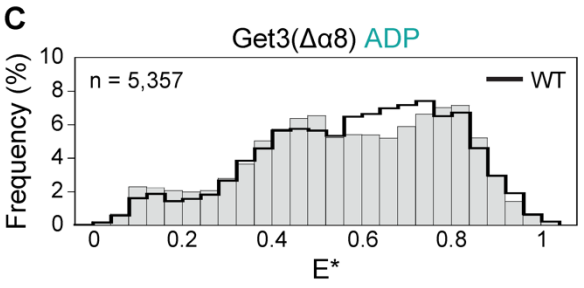
(D) Multi-turnover ATPase rate constants were determined with 0.5 μM mutant Get3(Δα8). The line was a fit of the data to Equation 9, which gave K_M and k_{cat} values of 2.2 ± 0.2 μM and 0.30 ± 0.02 min^{-1}, respectively. These values are within 2-fold to those previously reported for wild-type Get3 (K_M = 3.6 ± 1.0 μM and k_{cat} = 0.33 ± 0.03 min^{-1} at 0.5 μM Get3) (Rome et al., 2013).

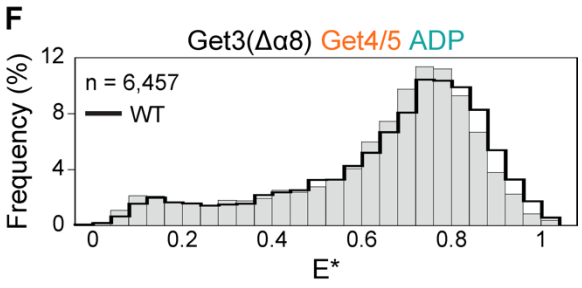
(E), (F) Deletion of α8 does not affect the binding of Get4/5 to Get3, nor the allosteric regulation of Get3 by ATP for Get4/5 binding. Equilibrium titrations were carried out to measure the binding of Get4/5 to wild-type (black) and mutant Get3(Δα8) (blue) in the apo (E) and ATP-bound (F) states. 0.5 μM of acrylodan-labeled Get4/5 was used in all experiments, and 2 mM ATP was present in experiments in (F). The lines are fits of the data to Equation 10, and gave K_d values of 3.8 ± 0.2 μM and 4.8 ± 3 μM for apo-Get3(WT) and Get3(Δα8), respectively, and 0.020 ± 0.008 μM and 0.015 ± 0.0003 μM for ATP-loaded Get3(WT) and Get3(Δα8), respectively. Values are reported as mean ± SD, with n = 2.

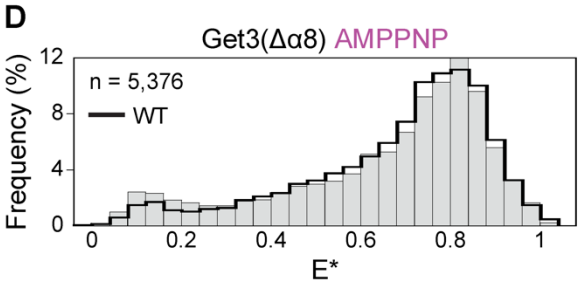

A
Get3 open
side view
Get3 closed
side view
Get3 open
top view
Get3 closed
top view
α8
α8
α8
~75 Å
Low FRET
~30 Å
High FRET


B
Get3(Δα8) apo
n = 8,283
WT
Frequency (%)
E*


E
Get3(Δα8) Get1CD
n = 5,166
WT
Frequency (%)
E*


C
Get3(Δα8) ADP
n = 5,357
WT
Frequency (%)
E*


F
Get3(Δα8) Get4/5 ADP
n = 6,457
WT
Frequency (%)
E*

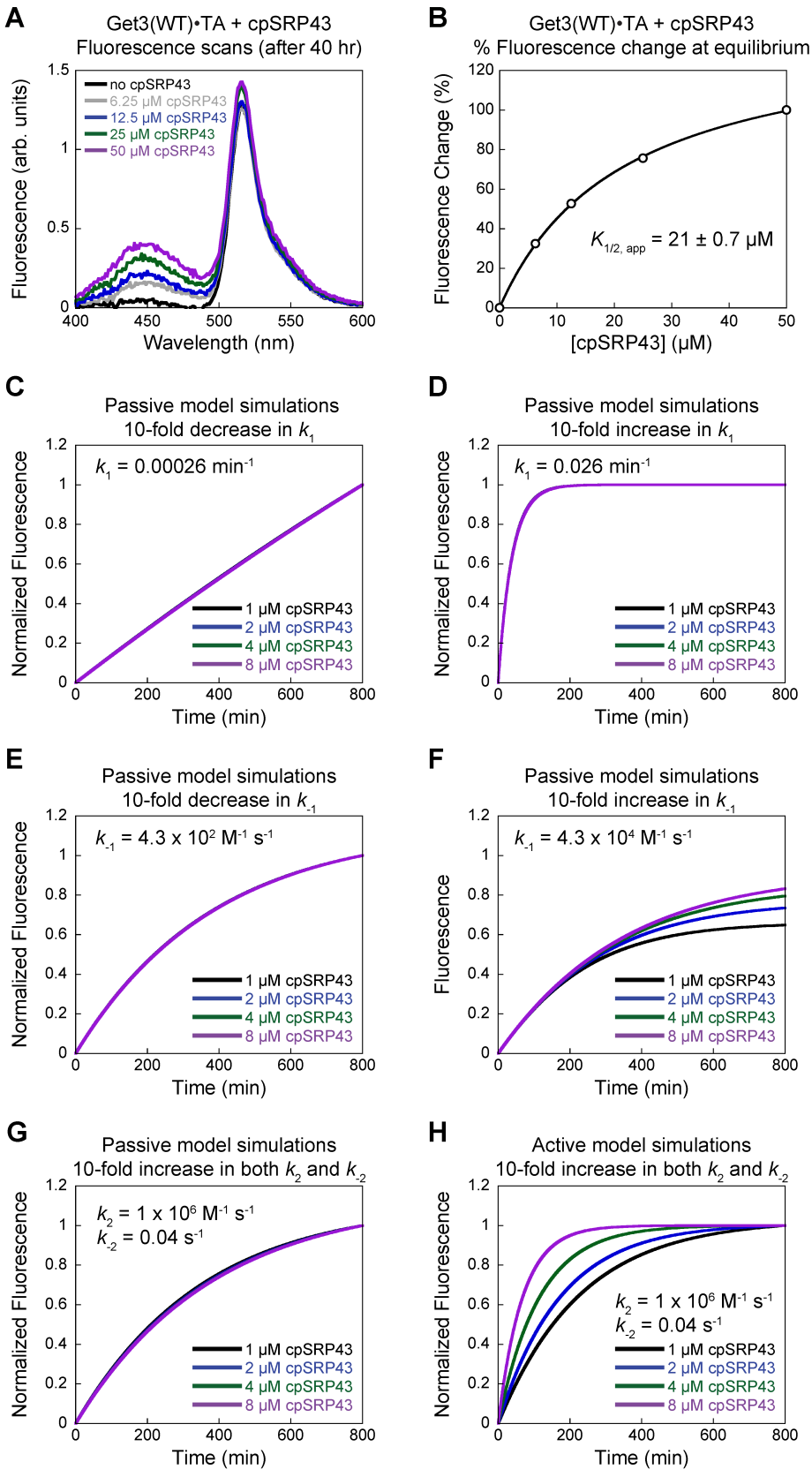

D
Get3(Δα8) AMPPNP
n = 5,376
WT
Frequency (%)
E*

100

Figure S3. Get3(Δα8) displays a similar conformational distribution and undergoes conformational regulations by nucleotides, Get1, and Get4/5 similarly to wild-type Get3. Related to Figures 1, 2, and 3.

(A) Approximate positions of donor and acceptor dyes (green and red stars, respectively) on Get3 in the *open* (left; PDB 3H84) and *closed* (right; PDB 2WOJ) conformations. The estimated distances between the dye pair are ~75 Å in the *open* conformation and ~30 Å in the *closed* conformation. The Förster radius of the Cy3B-ATTO 647N dye pair used in this work is ~55 Å. The approximate positions of α8 that were not resolved in the structures are highlighted as *cyan* circles in the bottom panels.

(B)-(F) FRET histograms illustrating conformational distributions for Get3(Δα8) in the apo (A), ADP-bound (B), AMPPNP-bound (C), Get1CD-bound (D), and Get4/5 and ADP-bound (E) states. The FRET histogram of Get3(Δα8) under each condition (grey bars) was similar to those displayed by wild-type Get3 (black lines, data adapted from (Chio et al., 2017b)). Importantly, Get3(Δα8) was specifically closed upon binding of AMPPNP (C) or binding with Get4/5 in the presence of ADP (E), and was opened by binding of Get1CD (D).


A
Get3(WT)•TA + cpSRP43
Fluorescence scans (after 40 hr)
Fluorescence (arb. units)
Wavelength (nm)
no cpSRP43
6.25 μM cpSRP43
12.5 μM cpSRP43
25 μM cpSRP43
50 μM cpSRP43
B
Get3(WT)•TA + cpSRP43
% Fluorescence change at equilibrium
Fluorescence Change (%)
[cpSRP43] (μM)
$K_{1/2, app}$ = 21 ± 0.7 μM
C
Passive model simulations
10-fold decrease in k_1
k_1 = 0.00026 min^{-1}
Normalized Fluorescence
Time (min)
1 μM cpSRP43
2 μM cpSRP43
4 μM cpSRP43
8 μM cpSRP43
D
Passive model simulations
10-fold increase in k_1
k_1 = 0.026 min^{-1}
E
Passive model simulations
10-fold decrease in k_{-1}
k_{-1} = 4.3 x 10^2 M^{-1} s^{-1}
F
Passive model simulations
10-fold increase in k_{-1}
k_{-1} = 4.3 x 10^4 M^{-1} s^{-1}
Fluorescence
G
Passive model simulations
10-fold increase in both k_2 and k_{-2}
k_2 = 1 x 10^6 M^{-1} s^{-1}
k_{-2} = 0.04 s^{-1}
H
Active model simulations
10-fold increase in both k_2 and k_{-2}

118

Figure S4. Additional kinetic simulations based on the passive and active models for TA release from Get3(Δα8). Related to Figure 1.

(A) Fluorescence emission spectra were recorded using an excitation wavelength of 370 nm and an emission wavelength of 450 nm 40 hours after 50 nM wild-type $Get3^{BDP}$•TA^{Cm} was chased with indicated concentrations of cpSRP43.

(B) The TA^{Cm} fluorescence change at equilibrium based on the data in (A) was plotted against cpSRP43 concentration. The data was fit to Equation 6, which gave a $K_{1/2,app}$ value of 21 ± 0.7 μM in the presence of 50 nM Get3. This suggests that TA is bound to Get3 by ~400-fold more strongly than to cpSRP43.

(C)-(F) Changes in Get3•TA dissociation and association kinetics do not qualitatively affect the conclusion that the passive model cannot explain the experimentally observed rate dependence on chase concentration in Figure 1B.

(G)-(H) Changes in the rate constants of cpSRP43•TA association and dissociation (the K_d value for cpSRP43•TA was held constant) do not affect the kinetic simulations for both the passive (E) and active (F) models.

Table S1. List of oligonucleotides used in this study.

Oligo name	Sequence	Oligo number
F-qc-Bos1 A228TAG	GCTAGTCTTTTGGATCTAGTTAATTCTCTTGATCATAGG	2571F
R-qc-Bos1 A228TAG	CCTATGATCAAGAGAATTAACTAGATCCAAAAGACTAGC	2571R
F-qc-pCAS Get3 T173	CGGGTGGCGAATGGGACTTTGTTGTAGAAATCTTAATGTG GTTTTAGAGCTAGAAATAGC	2609F
R-qc-pCAS Get3 T173	GCTATTTCTAGCTCTAAAACCACATTAAGATTTCTACAAC AAAGTCCCATTCGCCACCCG	2609R
F-qc-pCAS Get3 T342	CGGGTGGCGAATGGGACTTTGAATATAACCCTATTACTGA GTTTTAGAGCTAGAAATAGC	2610F
R-qc-pCAS Get3 T342	GCTATTTCTAGCTCTAAAACTCAGTAATAGGGTTATATTC AAAGTCCCATTCGCCACCCG	2610R
F-qc-Get3 T173 syn codon replacement	GACACTGCTCCAACTGGCCATACCCTGCGCTTCCTACAGC TACCAAATACTTTATCCAAG	2611F
R-qc-Get3 T173 syn codon replacement	CTTGGATAAAGTATTTGGTAGCTGTAGGAAGCGCAGGGT ATGGCCAGTTGGAGCAGTGTC	2611R
F-qc-Get3 T342 syn codon replacement	CTCACAGTTCCTAAACAAAGAGTACAATCCGATCACGGA TGGCAAAGTCATTTATGAG	2612F
R-qc-Get3 T342 syn codon replacement	CTCATAAATGACTTTGCCGTCCGTGATCGGATTGTACTCT TTGTTTAGGAACTGTGAG	2612R
F-amp-Get3 FLAG repair round 1	CAAGAAGATCATCACATTGTAATTATGGATTTAACCGTGG AACCTAATTTGCACTC	2613F
R-amp-Get3 FLAG repair round 1	GTATCTATTTATGGTATTCAGGGGCTTCTACTTGTCATCGT CGTCCTTGTAGTCGCCGG	2613R
F-amp-Get3 FLAG repair round 2	GCGTTTAGGAAAACGTACGACAAGAACAAGAAGATCATC ACATTGTAATTATGG	2614F
R-amp-Get3 FLAG repair round 2	TCTATGGTTATATGTCGTATGTATCTATTTATGGTATTCAG GGGCTTC	2614R